

PFOS:
A 96-HOUR STATIC ACUTE TOXICITY TEST WITH
THE SALTWATER MYSID (*Mysidopsis bahia*)

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-101
3M LAB REQUEST NO. U2723

U.S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1035

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STUDY COMPLETION DATE: June 24, 1999

AMENDED REPORT DATE: April 26, 2000

Submitted to

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St. Paul, MN 55144

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(410) 822-8600

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Static Acute Toxicity Test with the Saltwater Mysid (*Mysidopsis bahia*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-101

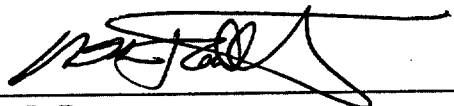
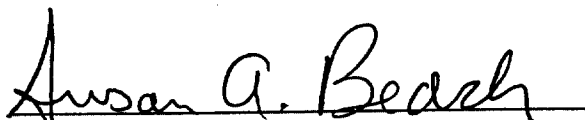
STUDY COMPLETION: June 24, 1999

AMENDED REPORT: April 26, 2000

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice, OCDE/GD (92) 32, Environment Monograph No. 45, Paris 1992; and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984, with the following exceptions:

The test substance was not characterized in accordance with full GLP compliance; however, the characterization was performed according to 3M Standard Operating Procedures and Methods, and all raw data are being maintained in the 3M archives. The test substance is being recharacterized in accordance with GLP.

The stability of the test substance under conditions of storage at the test site was not determined in accordance with Good Laboratory Practice Standards.

STUDY DIRECTOR:
Kurt R. Drott
Senior Biologist4/26/00
DATE**SPONSOR APPROVAL:**
Sponsor4/27/00
DATE**000935**

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QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice, OCDE/GD (92) 32, Environment Monograph No. 45, Paris 1992; and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO:	
		STUDY DIRECTOR:	MANAGEMENT:
Test Substance Preparation	March 5, 1999	March 8, 1999	March 9, 1999
Matrix Fortification and Sample Preparation	March 10, 1999	March 10, 1999	March 12, 1999
Biological Data and Draft Report	March 25, 1999	March 25, 1999	March 26, 1999
Analytical Data and Draft Report	March 24 and 25, 1999	March 25, 1999	March 25, 1999
Final Report	June 3, 1999	June 3, 1999	June 3, 1999
Amended Report	April 19, 2000	April 19, 2000	April 20, 2000

James H. Coleman
James H. Coleman
Quality Assurance Representative

4-20-00
DATE

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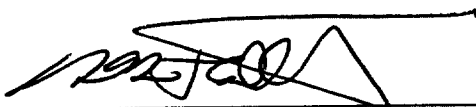
REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Static Acute Toxicity Test with the Saltwater Mysid
(*Mysidopsis bahia*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-101

STUDY DIRECTOR:



Kurt R. Drott
Senior Biologist

4/26/00

DATE

MANAGEMENT:



Henry O. Krueger, Ph.D.
Director, Aquatic Toxicology and
Non-Target Plants

4/26/00

DATE

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SUMMARY

SPONSOR:	3M Corporation
SPONSOR'S REPRESENTATIVE:	Ms. Susan A. Beach
LOCATION OF STUDY, RAW DATA AND A COPY OF THE FINAL REPORT:	Wildlife International Ltd. Easton, Maryland 21601

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER:	454A-101
TEST SUBSTANCE:	PFOS (Perfluorooctane Sulfonic Acid Potassium Salt)
STUDY:	PFOS: A 96-Hour Static Acute Toxicity Test with the Saltwater Mysid (<i>Mysidopsis bahia</i>)
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control; 0.57, 1.1, 1.9, 3.0 and 5.4 mg a.i./L
TEST DATES:	Experimental Start – March 8, 1999 Biological Termination – March 12, 1999 Experimental Termination – March 12, 1999
LENGTH OF TEST:	96 Hours

TEST ORGANISM:	Saltwater Mysid (<i>Mysidopsis bahia</i>)
SOURCE OF TEST ORGANISMS:	Wildlife International Ltd. cultures Easton, Maryland 21601
AGE OF TEST ORGANISMS:	Juveniles

96-HOUR LC50:	3.6 mg a.i./L
95% CONFIDENCE LIMITS:	3.0 and 4.6 mg a.i./L
NO MORTALITY CONCENTRATION:	1.1 mg a.i./L
NO-OBSERVED-EFFECT- CONCENTRATION:	1.1 mg a.i./L

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INTRODUCTION

This study was conducted by Wildlife International Ltd. for 3M Corporation at the Wildlife International Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from March 8, 1999 to March 12, 1999. Raw data generated by Wildlife International Ltd. and a copy of the final report are filed under Project Number 454A-101 in archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of this study was to evaluate the acute toxicity of PFOS (Perfluorooctane Sulfonic Acid Potassium Salt) to the saltwater mysid, *Mysidopsis bahia*, during a 96-hour exposure period under static test conditions.

EXPERIMENTAL DESIGN

Saltwater mysids were exposed to a geometric series of five test concentrations and a negative (saltwater) control. Two replicate test chambers were maintained in each treatment and control group, with 10 mysids in each test chamber for a total of 20 mysids per test concentration. Nominal test concentrations were selected in consultation with the Sponsor, and were based upon the results of an exploratory rangefinding toxicity test. Nominal test concentrations selected were 1.1, 1.8, 3.0, 4.9 and 8.2 mg active ingredient (a.i.)/L. Mean measured test concentrations were determined from samples of test water collected from each treatment and control group at the beginning of the test, at approximately 48 hours and at test termination.

The mysids were indiscriminately assigned to exposure chambers at test initiation. Observations of mortality and other clinical signs were made at approximately 4.5, 24, 48, 72, and 96 hours after test initiation. Cumulative percent mortality observed in the treatment groups was used to estimate or calculate LC50 values at 24, 48, 72 and 96 hours. The no mortality concentration and no-observed-effect-concentration (NOEC) were determined by visual interpretation of the mortality and clinical observation data.

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MATERIALS AND METHODS

The study was conducted based on the procedures outlined in the protocol, "PFOS: A 96-Hour Static Acute Toxicity Test with the Saltwater Mysid (*Mysidopsis bahia*)". The protocol was based on procedures outlined in U.S. Environmental Protection Agency Series 850 Ecological Effects Test Guidelines, OPPTS Number 850.1035 (1); U.S. Environmental Protection Agency, *Standard Evaluation Procedure, Acute Toxicity Test for Estuarine and Marine Organisms* (2); and ASTM Standard E729-88a *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3).

Test Substance

The test substance was received from 3M Corporation on October 29, 1998 and was assigned Wildlife International Ltd. identification number 4675. The test substance was described as a white powder. It was identified as FC-95 from lot number 217 (T-6295). Information provided by the Sponsor indicated a purity of 98.9%, and an expiration data of 2008. The test substance was reanalyzed by the Sponsor and the Certificate of Analysis dated March 9, 2000 indicated a purity of 90.49%. The test substance was stored at ambient room temperature.

Preparation of Test Concentrations

Nominal test concentrations were 1.1, 1.8, 3.0, 4.9 and 8.2 mg a.i./L, based on a test substance purity of 90.49%. All materials which came into contact with the test substance during preparation of test concentrations were constructed of plastic or stainless steel. A primary stock solution was prepared in dilution water at a concentration of 8.2 mg a.i./L. The primary stock solution was mixed with an electric mixer for approximately 22 hours to aid in the solubilization of the test substance. After mixing, the primary stock solution was proportionally diluted with dilution water to prepare the four additional test concentrations. All test solutions appeared clear and colorless.

Test Organism

The saltwater mysid, *Mysidopsis bahia*, was selected as the test species for this study. The saltwater mysid is representative of an important group of aquatic marine invertebrates and was selected for use in the test based upon past history of use and ease of culturing in the laboratory. Mysids used in the test were obtained as juveniles from cultures maintained by Wildlife International Ltd., Easton, Maryland.

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Adult mysids were held at approximately the same temperature as used during the test. During the holding and acclimation periods, the adults showed no signs of disease or stress. During the 14-day holding period preceding the test, water temperatures ranged from 25.0 to 25.5°C. The pH of the water ranged from 7.9 to 8.1, salinity was maintained at 20‰ (parts per thousand) and dissolved oxygen ranged from 7.0 to 7.2 mg/L. Instrumentation used for water measurements is described in the *Environmental Conditions* section of this report. At test initiation, the juvenile mysids were carefully collected from the cultures and transferred to glass beakers. Mysids were transferred from the beakers to the test compartments using a wide-bore pipette. Juvenile mysids are relatively fragile and were, therefore, handled as little as possible. The juvenile mysids were fed live brine shrimp (*Artemia* sp.) nauplii daily during the test to prevent cannibalism.

Test Apparatus

Test chambers were 2-L polyethylene buckets filled with approximately 1000 mL of test water. The depth of the test water in a representative test chamber was approximately 6.6 cm. Test chambers were impartially positioned in an environmental chamber designed to maintain a temperature of 25±2°C. The test chambers were labeled with the project number, test concentration and replicate and were covered with plexiglass throughout the test.

Dilution Water

The water used for holding, acclimation and testing was natural seawater collected at Indian River Inlet, Delaware, and diluted to a salinity of approximately 20‰ with well water. Salinity and pH measurements taken during the four-week period immediately preceding the test are presented in Appendix I.

The freshly-collected seawater was passed through a sand filter to remove particles greater than approximately 25 µm, and pumped into a 37,800-L storage tank. The filtered seawater then was diluted with freshwater from a well on the Wildlife International Ltd. site and aerated with spray nozzles. Prior to use, the water again was filtered (0.45 µm) to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in saltwater used by Wildlife International Ltd. are presented in Appendix II.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period

of low light intensity was provided when lights went on and off to avoid sudden changes in lighting. Light intensity at test initiation was approximately 437 lux at the surface of the water. Light intensity was measured using a SPER Scientific Ltd. light meter.

Temperature was measured in each test chamber at the beginning and end of the test using a liquid-in-glass thermometer. Temperature also was measured continuously in one negative control replicate using a Fulscope ER/C Recorder. The target test temperature during the study was $25 \pm 2^\circ\text{C}$. Dissolved oxygen and pH measurements were made on water samples collected from all replicate test chambers of each treatment and control at test initiation, and at approximately 24-hour intervals thereafter. Salinity was measured in the dilution water at test initiation.

Measurements of pH were made using a Fisher Accumet Model 915 pH meter, and dissolved oxygen was measured using a Yellow Springs Instrument Model 51B dissolved oxygen meter. Salinity was measured using a Bio-marine, Inc. Aquafauna refractometer.

Observations

Observations were made to determine the number of mortalities. The number of individuals exhibiting clinical signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 4.5, 24, 48, 72 and 96 hours after test initiation.

Statistical Analyses

The 24, 48, 72 and 96-hour LC50 values and the 95% confidence intervals were calculated when possible by probit analysis, the moving average method or binomial probability with non-linear interpolation (4,5,6) using the computer software of C.E. Stephan (7). In this study, LC50 values could not be calculated at 24 and 48 hours due to the lack of an adequate concentration-response pattern, however, the probit method was used to evaluate mortality at 72 and 96 hours. The no mortality concentration and NOEC were determined by visual interpretation of the mortality and clinical observation data.

Analytical Chemistry

Water samples were collected at mid-depth from each replicate test chamber of each treatment and control group at the beginning of the test, at 48 hours and at test termination to measure concentrations of the test substance. The samples were collected in plastic (Nalgene®) vials and analyzed as soon as possible without storage. Analytical procedures used in the analysis of the samples are provided in Appendix III.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Results of analyses to measure concentrations of PFOS in water samples collected during the test are presented in Table 1 and in the analytical chemistry report (Appendix III). Nominal concentrations selected for use in this study were 1.1, 1.8, 3.0, 4.9 and 8.2 mg a.i./L. Samples collected at test initiation had measured values that ranged from 52 to 71% of nominal values. After 22 hours of mixing, the primary stock solution was measured to be 70 to 71% of nominal and was probably at the limit of solubility in filtered saltwater. The recoveries in the other test concentrations were similar because they were proportional dilutions of the primary stock. Measured values for samples taken at 48 hours ranged from 44 to 71% of nominal. Measured values for samples taken at 96 hours ranged from 36 to 71% of nominal. When measured concentrations of the samples analyzed at test initiation, approximately 48 hours and at test termination were averaged, the mean measured concentrations for this study were 0.57, 1.1, 1.9, 3.0 and 5.4 mg a.i./L. Mean measured concentrations were used in the estimation or calculation of LC50 values.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH are presented in Table 2. Temperatures were within the $25 \pm 2^\circ\text{C}$ range established for the test. Dissolved oxygen concentrations remained ≥ 6.6 mg/L (90% of saturation) throughout the test. Measurements of pH ranged from 8.1 to 8.2 during the test.

Daily observations of mortality and other clinical signs of toxicity observed during the test are shown in Table 3. Mysids in the negative control, and the 0.57 and 1.1 mg a.i./L treatment groups appeared normal and healthy during the test. After 96-hours of exposure, mortality in the 1.9, 3.0 and 5.4 mg a.i./L treatment groups was 10, 40 and 75%, respectively. LC50 values and 95% confidence limits at 24, 48, 72 and 96 hours were estimated or calculated from the mortality data, and are shown in Table 4.

CONCLUSIONS

The 96-hour LC50 value for saltwater mysids exposed to PFOS was 3.6 mg a.i./L. The 95% confidence limits were 3.0 and 4.6 mg a.i./L, and the slope of the concentration-response curve was 4.5. The 96-hour no mortality concentration and the NOEC were 1.1 mg a.i./L.

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REFERENCES

- 1 U.S. Environmental Protection Agency. 1996. Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1035: *Mysid Acute Toxicity Test*.
- 2 U.S. Environmental Protection Agency. 1985. *Standard Evaluation Procedure, Acute Toxicity Test for Estuarine and Marine Organisms (Shrimp 96-Hour Acute Toxicity Test)*. Hazard Evaluation Division. Office of Pesticide Programs. EPA 540/9-85-010. Washington, D.C.
- 3 ASTM Standard E729-88a. 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians*. American Society for Testing and Materials.
- 4 Thompson, W.R. 1947. *Bacteriological Reviews*, Vol. II, No. 2. Pp. 115-145.
- 5 Stephan, C.E. 1977. "Methods for Calculating an LC50", *Aquatic Toxicology and Hazard Evaluations*. American Society for Testing and Materials. Publication Number STP 634, pp 65-84.
- 6 Finney, D.J. 1971. *Statistical Methods in Biological Assay*. Second edition. Griffin Press, London.
- 7 Stephan, C.E. 1978. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.

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Table 1

Summary of Analytical Chemistry Data

Sponsor:		3M Corporation			
Test Substance:		PFOS			
Test Organism:		Saltwater Mysid, <i>Mysidopsis bahia</i>			
Dilution Water:		Filtered Saltwater			
Nominal Test Concentration (mg a.i./L)	Replicate	Sampling Time (Hours)	Measured Concentration (mg a.i./L)	Mean Measured Concentration (mg a.i./L)	Percent Of Nominal
Negative Control	A	0	<LOQ	<LOQ	--
	B	0	<LOQ		
	A	48	<LOQ		
	B	48	<LOQ		
	A	96	<LOQ		
	B	96	<LOQ		
1.1	A	0	0.575	0.57	52
	B	0	0.622		
	A	48	0.605		
	B	48	0.640		
	A	96	0.391		
	B	96	0.580		
1.8	A	0	1.12	1.1	61
	B	0	1.19		
	A	48	1.10		
	B	48	1.09		
	A	96	1.04		
	B	96	1.13		
3.0	A	0	1.92	1.9	63
	B	0	1.99		
	A	48	1.92		
	B	48	1.91		
	A	96	1.79		
	B	96	1.91		
4.9	A	0	3.05	3.0	61
	B	0	2.66		
	A	48	2.96		
	B	48	3.35		
	A	96	3.11		
	B	96	3.11		
8.2	A	0	5.82	5.4	66
	B	0	5.78		
	A	48	3.58		
	B	48	5.85		
	A	96	5.22		
	B	96	5.86		

¹ The limit of quantitation (LOQ) was 0.115 mg a.i./L.

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Table 2

Temperature, Dissolved Oxygen and pH of Water in the Test Chambers

Sponsor:		3M Corporation											
Test Substance:		PFOS											
Test Organism:		Saltwater Mysid, <i>Mysidopsis bahia</i>											
Dilution Water:		Filtered Saltwater											
Mean Measured Test Concentration (mg a.i./L)	Replicate	0 Hour ¹			24 Hours		48 Hours		72 Hours		96 Hours		
		Temp ² (°C)	DO ³ (mg/L)	pH	DO (mg/L)	pH	DO (mg/L)	pH	DO (mg/L)	pH	Temp (°C)	DO (mg/L)	pH
Negative Control	A	24.2	7.4	8.1	7.1	8.1	6.9	8.2	6.8	8.1	24.7	6.8	8.2
	B	25.4	7.2	8.1	7.0	8.1	6.8	8.2	6.8	8.1	24.7	6.8	8.2
0.57	A	24.1	7.4	8.1	7.0	8.1	6.8	8.2	6.8	8.1	24.7	6.7	8.2
	B	25.3	7.2	8.2	7.0	8.1	6.8	8.2	6.8	8.2	24.7	6.7	8.2
1.1	A	24.0	7.4	8.2	7.0	8.1	6.8	8.2	6.8	8.1	24.7	6.8	8.2
	B	24.4	7.3	8.2	7.0	8.1	8.8	8.2	6.8	8.2	24.8	6.7	8.2
1.9	A	24.0	7.4	8.2	7.1	8.1	6.9	8.2	6.8	8.2	24.7	6.6	8.2
	B	23.8	7.2	8.2	7.0	8.1	6.8	8.2	6.8	8.1	24.6	6.6	8.2
3.0	A	23.6	7.3	8.2	7.0	8.1	6.8	8.2	6.7	8.2	24.5	6.8	8.2
	B	23.5	7.4	8.2	7.0	8.1	6.9	8.2	6.8	8.2	24.4	6.8	8.2
5.4	A	23.8	7.3	8.2	7.1	8.1	6.8	8.2	6.8	8.2	24.5	6.9	8.2
	B	24.0	7.2	8.2	7.0	8.1	6.9	8.2	6.8	8.2	24.5	6.8	8.2

¹ The 0-hour dilution water (negative control) measurement for salinity 20‰.² Temperature measured continuously during the test ranged from approximately 23.5 to 26.0°C.³ A dissolved oxygen concentration of 4.4 mg/L represents 60% saturation at 25°C in saltwater with a salinity of 20‰.

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Table 3

Cumulative Percent Mortality and Treatment-Related Effects

Sponsor:		3M Corporation															
Test Substance:		PFOS															
Test Organism:		Saltwater Mysid, <i>Mysidopsis bahia</i>															
Dilution Water:		Filtered Saltwater															
Mean Measured Test Concentration (mg a.i./L)	Replicate	No. Exposed	4.5 Hours			24 Hours			48 Hours			72 Hours			96 Hours		Cumulative Percent Mortality
			No. Dead ¹	Effects ²	% Dead	No. Dead	Effects	% Dead	No. Dead	Effects	% Dead	No. Dead	Effects	% Dead	No. Dead	Effects	
Negative Control	A	10	0	10 AN	0	0	10 AN	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
	B	10	0	10 AN		0	10 AN		0	10 AN		0	10 AN		0	10 AN	
0.57	A	10	0	10 AN	0	0	10 AN	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
	B	10	0	10 AN		0	10 AN		0	10 AN		0	10 AN		0	10 AN	
1.1	A	10	0	10 AN	0	0	10 AN	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
	B	10	0	10 AN		0	10 AN		0	10 AN		0	10 AN		0	10 AN	
1.9	A	10	0	10 AN	0	0	10 AN	0	0	10 AN	0	0	10 AN	5	0	10 AN	10
	B	10	0	10 AN		0	10 AN		0	10 AN		1	9 AN		2	8 AN	
3.0	A	10	0	10 AN	0	0	10 AN	5	2	8 AN	15	4	6 AN	30	5	5 AN	40
	B	10	0	10 AN		1	9 AN		1	9 AN		2	8 AN		3	7 AN	
5.4	A	10	0	10 AN	0	2	8 AN	15	4	6 AN	45	6	4 AN	60	8	2 AN	75
	B	10	0	10 AN		1	9 AN		5	5 AN		6	4 AN		7	3 AN	

¹ Cumulative number of dead mysids.² Observed Effects: AN = Appears Normal.

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Table 4
LC50 Values

Sponsor:	3M Corporation			
Test Substance:	PFOS			
Test Organism:	Saltwater Mysid, <i>Mysidopsis bahia</i>			
Dilution Water:	Filtered Saltwater			
Time	LC50 (mg a.i./L)	Lower 95% Confidence Limits (mg a.i./L)	Upper 95% Confidence Limits (mg a.i./L)	Statistical Method
24 Hours	> 5.4	-- ¹	-- ¹	Visual Interpretation
48 Hours	> 5.4	-- ¹	-- ¹	Visual Interpretation
72 Hours	4.4	3.6	6.2	Probit
96 Hours	3.6	3.0	4.6	Probit
¹ Confidence limits could not be calculated with the mortality data obtained.				

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APPENDIX I

Salinity and pH of Saltwater Measured During the 4-Week
Period Immediately Preceding the Test

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Saltwater Mysid, <i>Mysidopsis bahia</i>
Dilution Water:	Filtered Saltwater

	Mean	Range
Salinity (‰)	20 (N = 4)	20 – 20
pH	8.2 (N = 4)	8.1 – 8.2

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APPENDIX II
Analyses of Pesticides, Organics, Metals and Other Inorganics
in Wildlife International Ltd. Saltwater¹

ANALYSIS		MEASURED CONCENTRATION	
Miscellaneous Measurements			
Total Dissolved Solids		23,500	mg/L
Ammonia Nitrogen	<	0.050	mg/L
Total Organic Carbon ²	<	1.0	mg/L
Total Cyanide	<	10.0	µg/L
Organochlorines and PCBs			
Aldrin	<	0.005	µg/L
Alpha BHC	<	0.005	µg/L
Beta BHC	<	0.005	µg/L
Delta BHC	<	0.005	µg/L
Gamma BHC (Lindane)	<	0.006	µg/L
Chlordane	<	0.025	µg/L
DDD, pp'	<	0.006	µg/L
DDE, pp'	<	0.005	µg/L
DDT, pp'	<	0.008	µg/L
Dieldrin	<	0.005	µg/L
Endosulfan, A	<	0.005	µg/L
Endosulfan, B	<	0.005	µg/L
Endosulfan Sulfate	<	0.018	µg/L
Endrin	<	0.010	µg/L
Endrin Aldehyde	<	0.005	µg/L
Heptachlor	<	0.005	µg/L
Methoxychlor	<	0.007	µg/L
Heptachlor Epoxide	<	0.005	µg/L
Toxaphene	<	0.500	µg/L
PCB-1016	<	0.260	µg/L
PCB-1221	<	0.260	µg/L
PCB-1232	<	0.260	µg/L
PCB-1242	<	0.720	µg/L
PCB-1248	<	0.720	µg/L
PCB-1254	<	0.720	µg/L
PCB-1260	<	0.720	µg/L
Metals and Other Inorganics			
Aluminum ³	<	100	µg/L
Arsenic ³	<	25.0	µg/L
Beryllium ³	<	0.50	µg/L
Cadmium ³	<	1.0	µg/L
Calcium ³		235	mg/L
Chromium ³	<	2.0	µg/L
Cobalt ³	<	1.0	µg/L
Copper ³	<	20.0	µg/L
Iron ³	<	100	µg/L
Lead ³	<	10.0	µg/L
Magnesium ³		760	mg/L
Manganese ³	<	4.0	µg/L
Mercury ³	<	0.20	µg/L
Molybdenum ³	<	2.0	µg/L
Nickel ³	<	20.0	µg/L
Potassium ³		277	mg/L
Selenium ³	<	25.0	µg/L
Silver ³	<	1.0	µg/L
Sodium ³		6,010	mg/L
Zinc ³	<	20.0	µg/L

¹ Analyses performed by QST Environmental, Gainesville, Florida for samples collected on November 3 through November 7, 1997.² Analyses performed by Wildlife International Ltd. for the sample collected on November 5, 1997.³ Analyses performed by Wildlife International Ltd. for samples collected on November 5 through 7, 1997.

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APPENDIX III

THE ANALYSIS OF PFOS IN FILTERED SALTWATER
IN SUPPORT OF
WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 454A-101

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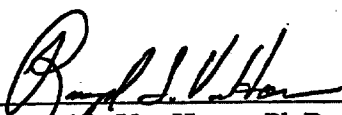
REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Static Acute Toxicity Test with the Saltwater Mysid
(*Mysidopsis bahia*)

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 454A-101

PRINCIPAL INVESTIGATOR:



Raymond L. Van Hoven, Ph.D.
Scientist

4-20-00

DATE

MANAGEMENT:



Willard B. Nixon, Ph.D.
Manager, Analytical Chemistry

4/20/00

DATE

000954

AMENDED

Introduction

Saltwater samples were collected from a static acute aquatic toxicity study designed to determine the effects of PFOS (Perfluorooctane Sulfonic Acid Potassium Salt) to the saltwater mysid (*Mysidopsis bahia*). This study was conducted by Wildlife International Ltd. and identified as Project No.: 454A-101. The analyses of these water samples were performed at Wildlife International Ltd. using high performance liquid chromatography with mass spectrometric detection (HPLC/MS). Samples were received for analysis on March 8, 10 and 12, 1999 and were analyzed on each sample receipt day.

Test Substance and Internal Standard

The test substance used for the analytical portion of this study was Wildlife International Ltd. identification number 4675. The test substance was used to prepare calibration and matrix fortification samples.

The internal standard was received from 3M Corporation on July 2, 1998 and was assigned Wildlife International Ltd. identification number 4526 upon receipt. The internal standard, a granular material, was identified as: 1H, 1H, 2H, 2H Perfluorooctane Sulfonic Acid, Chemical Abstract Number: 27619-97-2. The standard was stored under ambient conditions.

Analytical Method

The method used for the analysis of the saltwater samples was developed at Wildlife International Ltd. and entitled "Analytical Method for the Determination of PFOS in Freshwater, Saltwater, and Algal Medium". This methodology was included as Appendix II of Wildlife International Ltd. protocol number 454/011299/MVAL/SUB454. It was based upon methodology provided by 3M Corporation.

Samples were diluted in a 50% methanol : 50% NANOpure® water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v) so that they fell within the calibration range of the PFOS methodology.

Concentrations of the PFOS in the standards and samples were determined by reverse-phase high performance liquid chromatography using a Hewlett-Packard Model 1100 High Performance Liquid

Chromatograph (HPLC) with a Perkin-Elmer API 100LC Mass Spectrometer equipped with a Perkin-Elmer TurboIonSpray ion source. HPLC separations were achieved using a Keystone Betasil C₁₈ analytical column (100 mm x 2 mm I.D., 3 µm particle size). The instrument parameters are summarized in Table 1. A method flowchart is provided in Figure 1.

Calibration Curve and Limit of Quantitation

Calibration standards of PFOS prepared in a 50% methanol : 50% NANOpure® water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v), ranging in concentration from 0.00229 to 0.0457 mg a.i./L, were analyzed with the samples. Linear regression equations were generated using peak area response ratios (PFOS : internal standard) versus the respective concentrations of the calibration standards. A typical calibration curve is presented in Figure 2. The concentration of PFOS in the samples was determined by substituting the peak area response ratios into the applicable linear regression equation. Representative ion chromatograms of low and high calibration standards are presented in Figures 3 and 4, respectively.

The method limit of quantitation (LOQ) for these analyses was set at 0.115 mg a.i./L calculated as the product of the lowest calibration standard analyzed (0.00229 mg a.i./L) and the dilution factor of the matrix blank samples (50.0).

Matrix Blank and Fortification Samples

Three matrix blank samples were analyzed to determine possible interference. No interferences were observed at or above the LOQ during samples analyses (Table 2). A representative chromatogram of a matrix blank is presented in Figure 5.

Saltwater was fortified at 0.457, 4.57 and 9.15 mg a.i./L and analyzed concurrently with the samples to determine the mean procedural recovery (Table 3). Sample concentrations were not corrected for the mean procedural recovery of 97.4%. A representative chromatogram of a matrix fortification is presented in Figure 6.

000956

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Example Calculations

Sample number 454A-101-3, nominal concentration of 1.1 mg a.i./L in saltwater.

Initial Volume: 0.500 mL

Final Volume: 25.0 mL

Dilution Factor: 50.0

PFOS Peak Area: 235466

Internal Standard Peak Area: 449012

Peak Area Ratio: 0.5244

Calibration curve equation.

Slope: 0.04044

Intercept: 0.05941

Curve is weighted (1/x).

$$\text{PFOS } (\mu\text{g a.i./L}) \text{ at instrument} = \frac{\text{Peak area ratio} - (\text{Y-intercept})}{\text{Slope}}$$

$$= \frac{0.5244 - 0.05941}{0.04044}$$

$$= 11.5$$

$$\text{PFOS (mg a.i./L) in sample} = \frac{\text{PFOS } (\mu\text{g a.i./L}) \text{ at instrument} \times \text{Dilution Factor}}{1000}$$

$$= \frac{11.5 \times 50.0}{1000}$$

$$= 0.575$$

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$$\text{Percent of Nominal Concentration} = \frac{\text{PFOS (mg a.i./L) in sample}}{\text{PFOS (mg a.i./L) nominal}} \times 100$$

Calculated recovery: 52.4%

Note: manual calculation may differ.

RESULTS

Sample Analysis

Saltwater samples were collected from the static acute toxicity study with the saltwater mysid (*Mysidopsis bahia*) at test initiation, March 8, 1999 (Hour 0), on March 10, 1999 (Hour 48), and at test termination, March 12, 1999 (Hour 96). The measured concentrations of PFOS in the samples collected at initiation of exposure of the test organisms (Hour 0) ranged from 52.4 to 70.7% of the nominal concentrations. Samples collected at Hour 48 had a measured concentration range of 43.5 to 71.0% of nominal values. Samples collected at test termination (Hour 96) had a measured concentration range of 35.5 to 71.1% of nominal values (Table 4). A representative chromatogram of a test sample is shown in Figure 7.

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Table 1

Typical HPLC Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a Perkin-Elmer API 100LC Mass Spectrometer equipped with a Perkin-Elmer TurboIonSpray ion source. Operated in selective ion monitoring mode (SIM).
ANALYTICAL COLUMN:	Keystone Betasil C ₁₈ column (100 mm x 2 mm I.D., 3 µm particle size)
OVEN TEMPERATURE:	30°C
STOP TIME:	10.0 minutes
FLOW RATE:	0.220 mL/minute
MOBILE PHASE:	72.0% Methanol : 28.0% NANOpure® Water containing 0.1% Formic Acid
INJECTION VOLUME:	25.0 µL
PFOS RETENTION TIME:	Approximately 7.2 minutes
INTERNAL STANDARD RETENTION TIME:	Approximately 4.9 minutes
PFOS MONITORED MASS:	498.6 amu
INTERNAL STANDARD MONITORED MASS:	426.7 amu

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Table 2

Matrix Blanks Analyzed Concurrently During Sample Analysis

Sample		Measured Concentration of PFOS ¹ (mg a.i./L)
Number (454A-101-)	Type	
MAB-1	Matrix Blank	< LOQ
MAB-2	Matrix Blank	< LOQ
MAB-3	Matrix Blank	< LOQ

¹ The limit of quantitation (LOQ) was 0.115 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00229 mg a.i./L) and the dilution factor of the matrix blank samples (50.0).

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Table 3

Matrix Fortifications Analyzed Concurrently During Sample Analysis

Sample Number (454A-101-)	Concentrations of PFOS (mg a.i./L)		Percent Recovered
	Fortified	Measured	
MAS-1	0.457	0.537	117
MAS-4	0.457	— ¹	--
MAS-7	0.457	0.396	86.5
MAS-2	4.57	4.27	93.3
MAS-5	4.57	4.43	96.8
MAS-8	4.57	4.36	95.2
MAS-3	9.15	8.18	89.4
MAS-6	9.15	9.42	103
MAS-9	9.15	8.93	97.6
			Mean = 97.4
			Standard Deviation = 9.41
			CV = 9.67
			N = 8

Note: Results and corrections for new test substance purity were generated using MacQuan version 1.5 software and manual calculations. Values have been rounded for reporting purposes.

¹ Result was 0.942 mg a.i./L or 205% of the nominal concentration. This result was not used to determine the mean procedural recovery due to probable fortification and/or dilution error.

000961

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Table 4

Measured Concentrations of PFOS in Saltwater Samples from a
Saltwater Mysid Static Acute Toxicity Test

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-101-)	Sampling Time (Hours)	PFOS Measured Concentration ¹ (mg a.i./L)	Percent of Nominal
0.0	1	0	< LOQ	--
	2	0	< LOQ	--
	13	48	< LOQ	--
	14	48	< LOQ	--
	25	96	< LOQ	--
	26	96	< LOQ	--
1.1	3	0	0.575	52.4
	4	0	0.622	56.6
	15	48	0.605	55.1
	16	48	0.640	58.2
	27	96	0.391	35.5
	28	96	0.580	52.9
1.8	5	0	1.12	64.4
	6	0	1.19	68.3
	17	48	1.10	63.2
	18	48	1.09	62.7
	29	96	1.04	59.8
	30	96	1.13	64.5
3.0	7	0	1.92	65.7
	8	0	1.99	67.9
	19	48	1.92	65.8
	20	48	1.91	65.2
	31	96	1.79	61.2
	32	96	1.91	65.4

Note: Results and corrections for new test substance purity were generated using MacQuan version 1.5 software and manual calculations. Values have been rounded for reporting purposes.

¹The limit of quantitation (LOQ) was 0.115 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00229 mg a.i./L) and the dilution factor of the matrix blank samples (50.0).

²Mean result of duplicate redilutions of the original sample. Original result (12.0 mg a.i./L) was not included in the statistical analysis of the data.

Duplicate results for 454A-101-23 are 3.55 and 3.61.

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Table 4 (Continued)

Measured Concentrations of PFOS in Saltwater Samples from a
Saltwater Mysid Static Acute Toxicity Test

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-101-)	Sampling Time (Hours)	PFOS Measured Concentration ¹ (mg a.i./L)	Percent of Nominal
4.9	9	0	3.05	61.6
	10	0	2.66	53.8
	21	48	2.96	59.9
	22	48	3.35	67.8
	33	96	3.11	63.0
	34	96	3.11	62.9
8.2	11	0	5.82	70.7
	12	0	5.78	70.2
	23	48	3.58 ²	43.5
	24	48	5.85	71.0
	35	96	5.22	63.3
	36	96	5.86	71.1

Note: Results and corrections for new test substance purity were generated using MacQuan version 1.5 software and manual calculations. Values have been rounded for reporting purposes.

¹The limit of quantitation (LOQ) was 0.115 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00229 mg a.i./L) and the dilution factor of the matrix blank samples (50.0).

²Mean result of duplicate redilutions of the original sample. Original result (12.0 mg a.i./L) was not included in the statistical analysis of the data.

Duplicate results for 454A-101-23 are 3.55 and 3.61.

000963

AMENDED

**METHOD OUTLINE FOR THE ANALYSIS OF PFOS
IN SALTWATER**

Prepare matrix fortification samples by spiking the requisite volume of PFOS stock solutions directly into filtered saltwater using gas-tight syringes and Class A volumetric flasks.



Dilute matrix fortification and test samples into the range of the calibration standards by partially filling Class A volumetric flasks with 50% methanol : 50% NANOpure® water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v). Add the appropriate volume of sample and bring the flask to volume with the dilution solvent. Process the matrix blank sample using the same dilution and aliquot volume as for the lowest fortification level. Mix well by several repeat inversions.



Ampulate samples and submit for LCMS analysis.

Figure 1. Analytical method flowchart for the analysis of PFOS in saltwater.

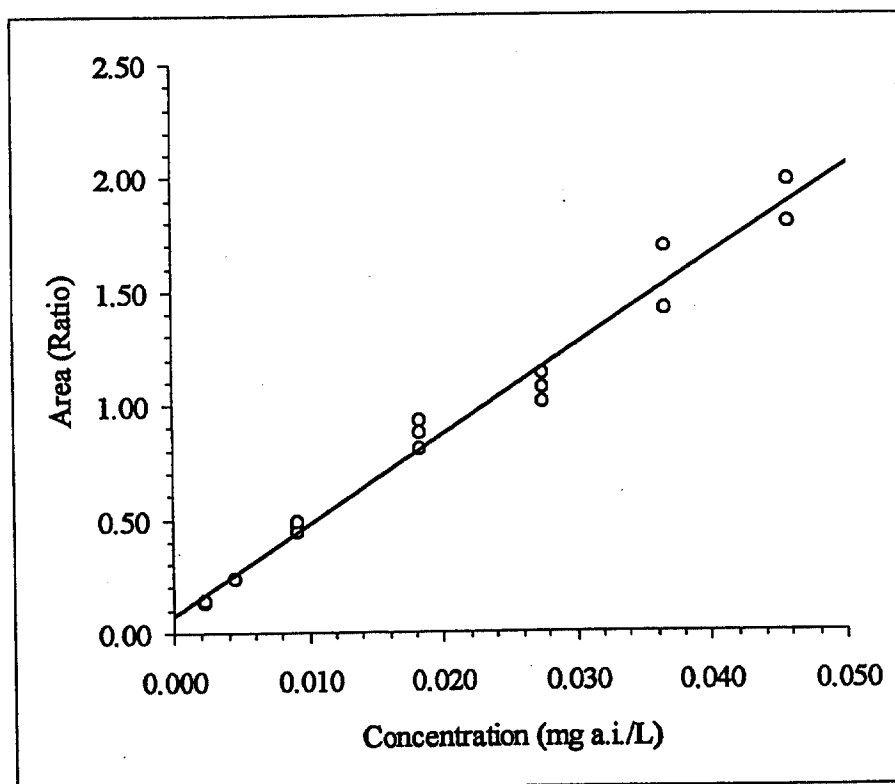


Figure 2. A typical calibration curve for PFOS. Slope = 0.04044; Intercept = 0.05941; $r = 0.9930$.
Curve is weighted ($1/x$).

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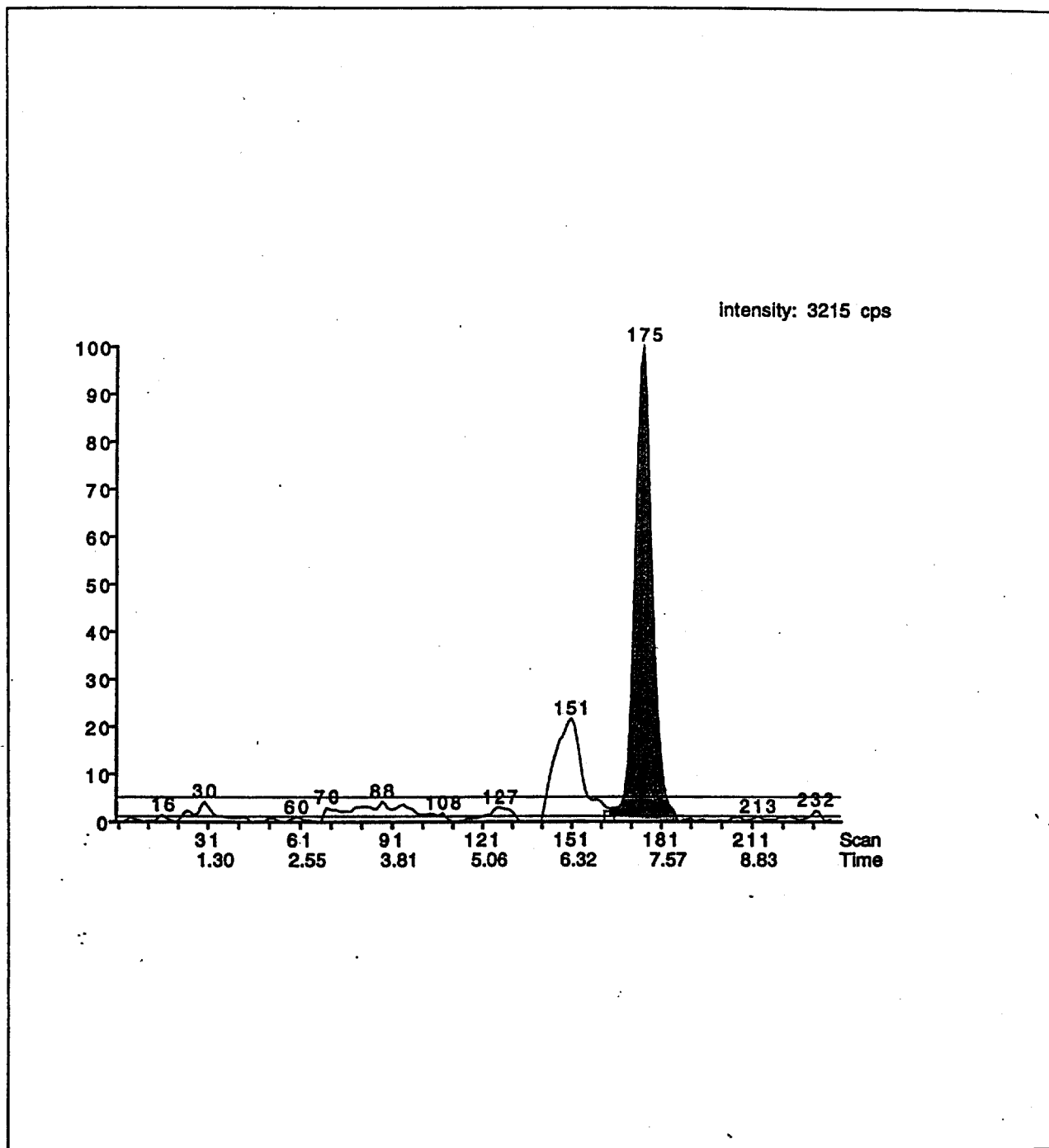


Figure 3. A representative ion chromatogram of a low-level (0.00229 mg a.i./L) PFOS standard.

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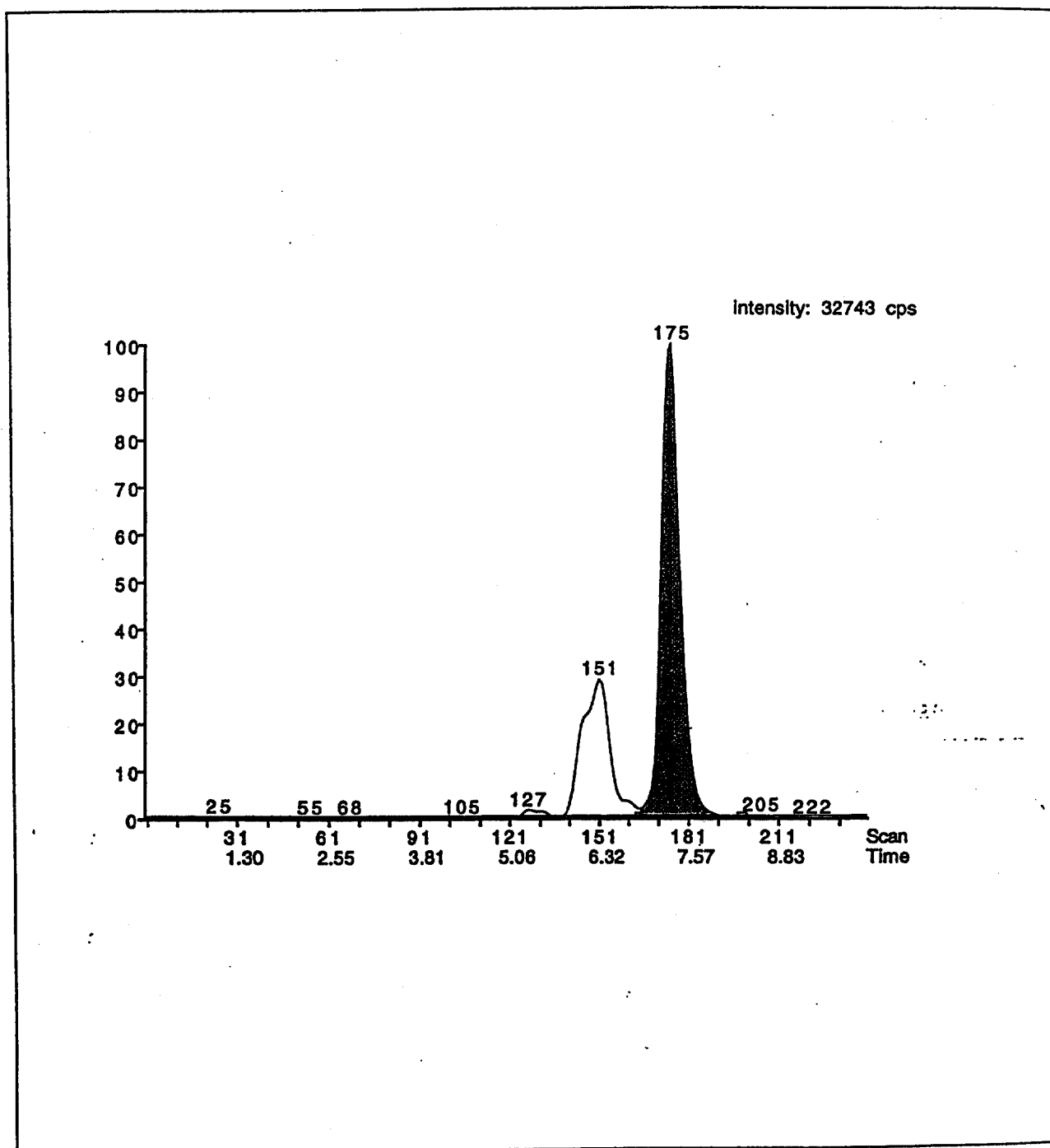


Figure 4. A representative ion chromatogram of a high-level (0.0457 mg a.i./L) PFOS standard.

000967

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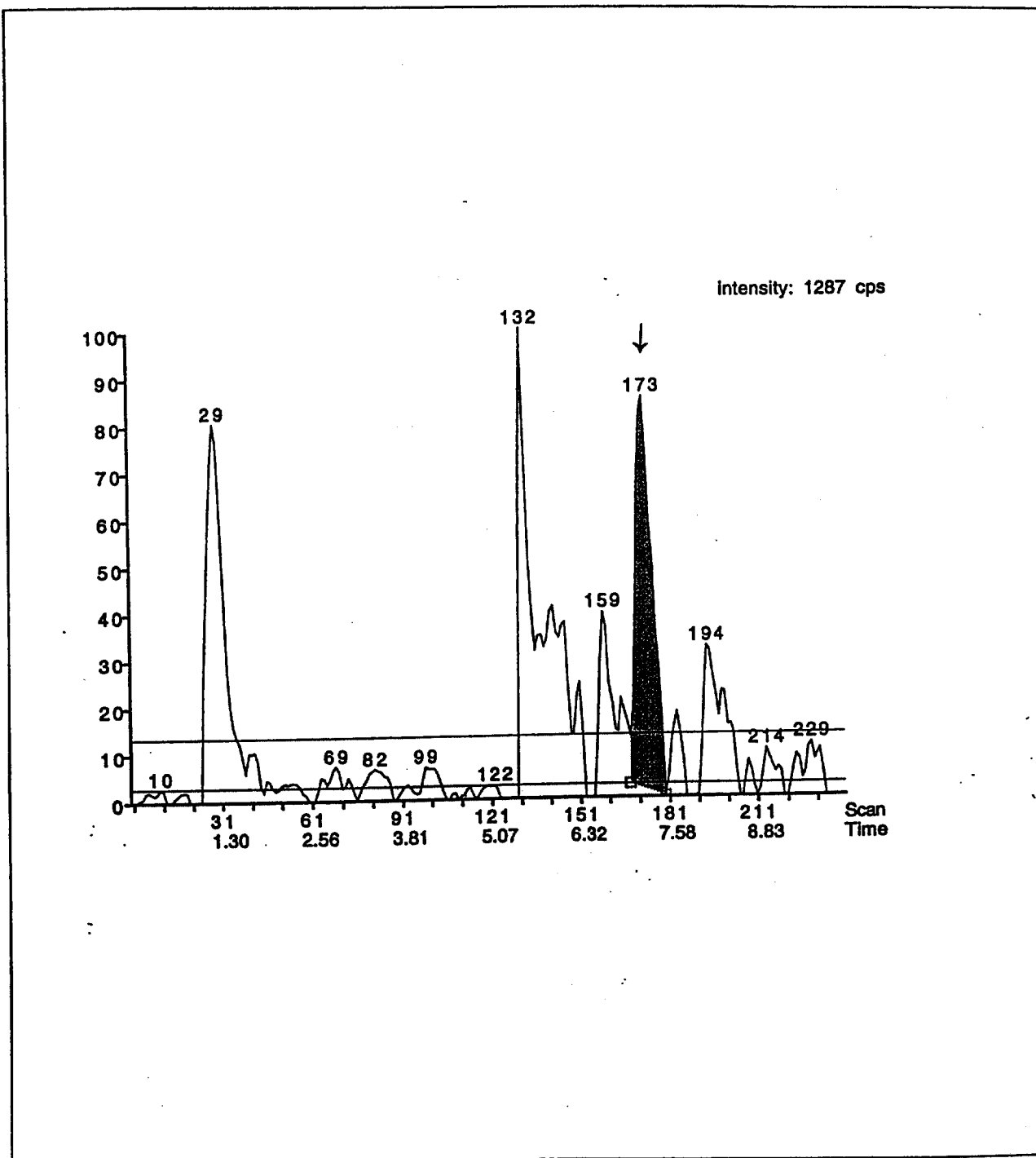


Figure 5. A representative chromatogram of a matrix blank sample (454A-101-MAB-1).
The arrow indicates the retention time of PFOS.

000968

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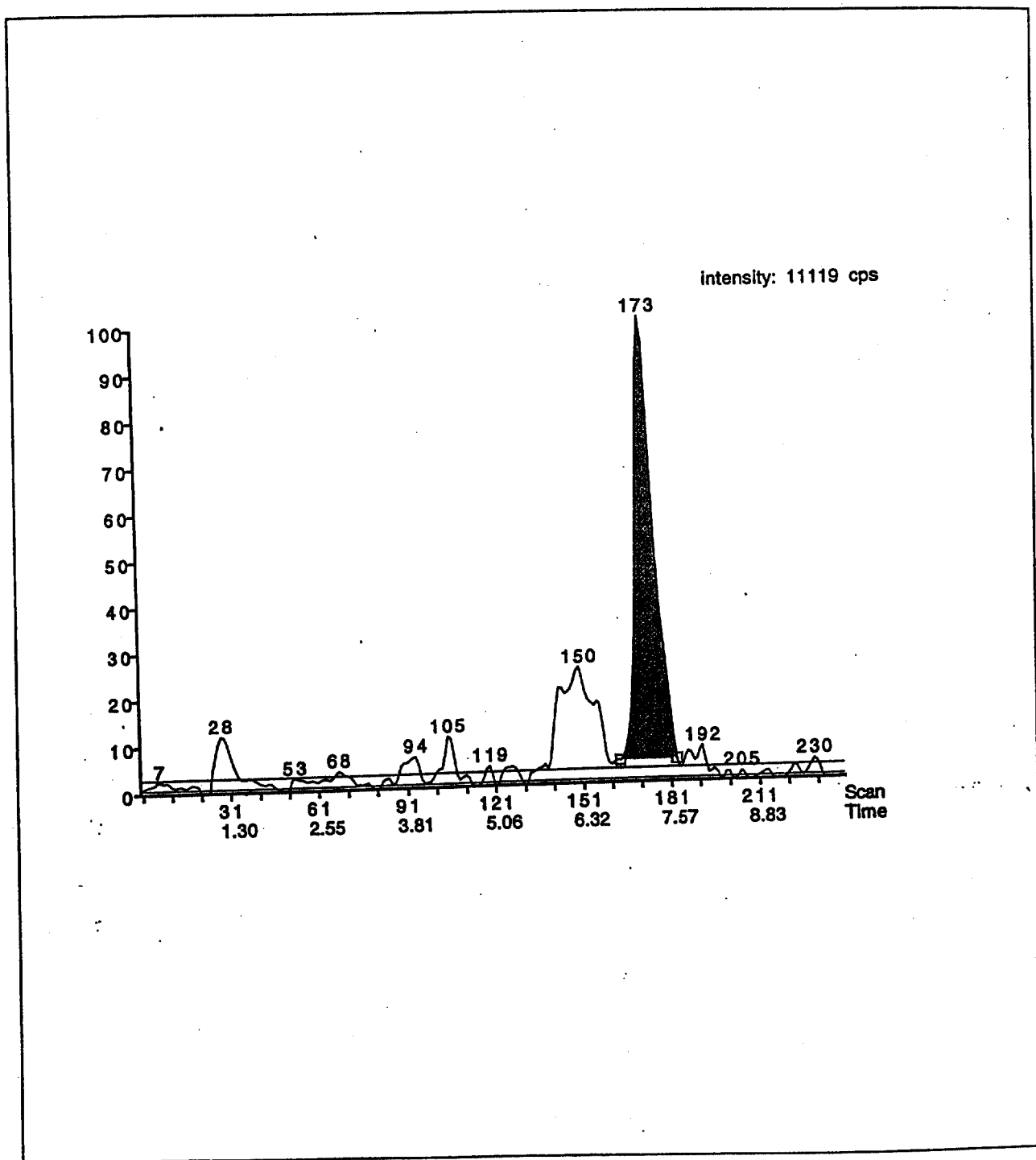


Figure 6. A representative chromatogram of a matrix fortification sample (454A-101-MAS-1).

000969

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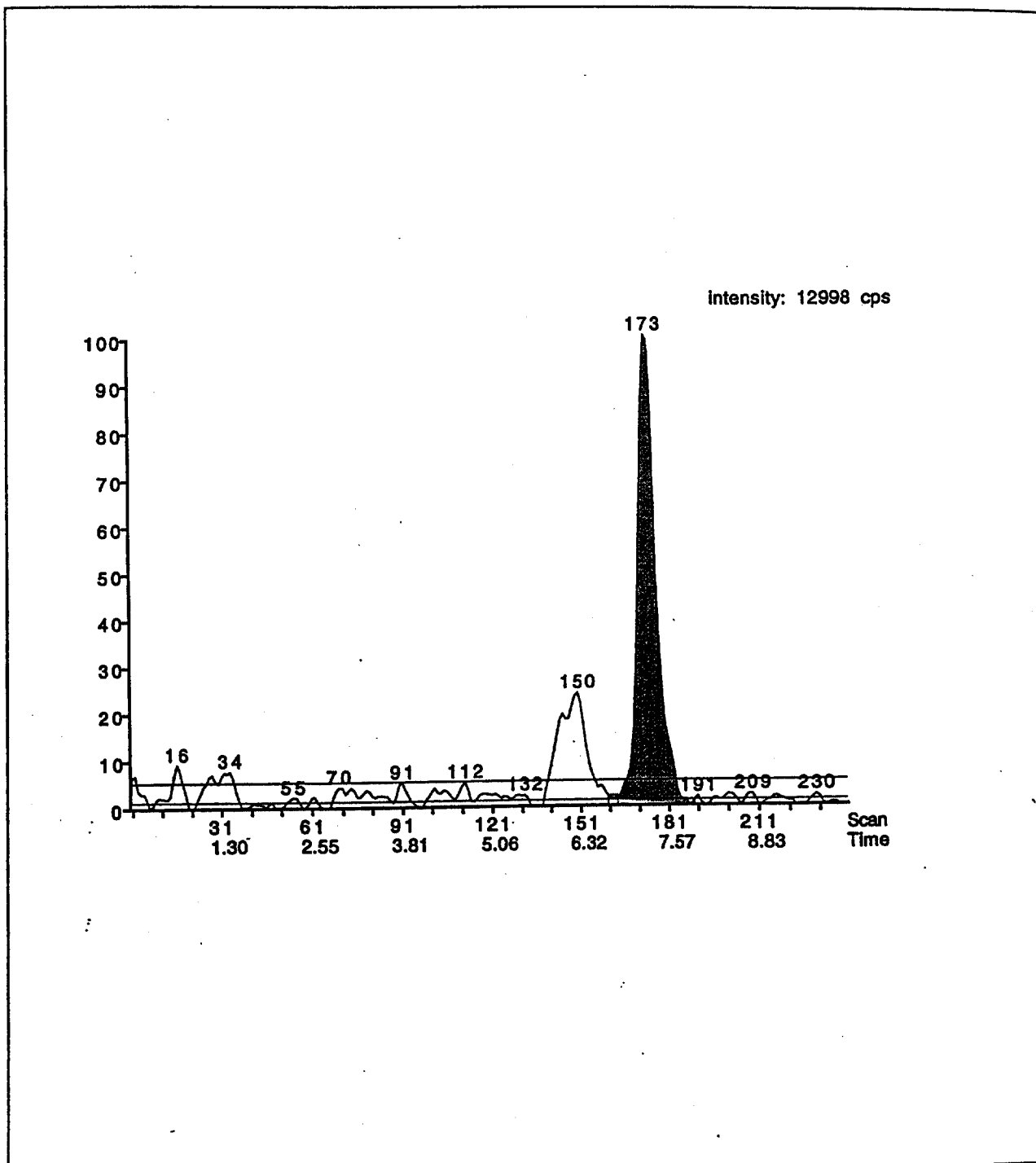


Figure 7. A representative chromatogram of a test sample (454A-101-3).

000970

APPENDIX IV

Changes to Protocol

This study was conducted in accordance with the approved Protocol with the following changes:

1. The protocol was amended to add the proposed experimental start and termination dates, test substance information and test concentrations.
2. The protocol was amended to remove annual feed analysis.
3. The protocol was amended to specify the analytical method to be used.
4. The test chambers were plastic buckets.
5. pH was measured daily in all test chambers.

APPENDIX V

Personnel Involved in the Study

The following key Wildlife International Ltd. personnel were involved in the conduct or management of this study:

1. Henry O. Krueger, Ph.D., Director, Aquatic Toxicology and Non-Target Plants
2. Willard B. Nixon, Ph.D., Manager, Analytical Chemistry
3. Mark A. Mank, Laboratory Supervisor
4. Timothy Z. Kendall, Laboratory Supervisor
5. Kurt R. Drott, Senior Biologist
6. Raymond L. VanHoven, Ph.D., Scientist

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APPENDIX VI

Report Amendment

1. Original Report: Pages 1-4, 6 and 21
Amendment: The pages were changed to include the amended report date, revised page numbers, and new signatures and dates due to the addition of the report amendment as Appendix VI.
Reason: To reflect the issuing of an amended report.
2. Original Report: Page 2
Amendment: The compliance statement was revised.
Reason: To clarify how the test substance was characterized.
3. Original Report: Page 9
Amendment: Information provided by the Sponsor reflecting the reanalysis of the test substance, including the reanalysis date and the purity, was added to the Test Substance section.
Reason: To reflect the current test substance information provided by the Sponsor.
4. Original Report: Entire report
Amendment: All test substance concentrations were changed to reflect the purity of the test substance as determined by the Sponsor in a reanalysis of the test substance (FC-95, Lot 217). Test concentrations originally were based on the reported purity of 98.9%. The certificate of analysis dated March 9, 2000 indicated a purity of 90.49%. Therefore, all test substance concentrations, including nominal concentrations, measured concentrations, and LC50 values, were recalculated and reported as mg a.i./L based on the 90.49% purity.
Reason: To report the results of the test based on the test substance purity of 90.49% at the request of the Sponsor.
5. Original Report: Page 7
Amendment: Experimental Termination – March 12, 1999
Reason: To report the experimental termination date for the study.

000973

AMENDED

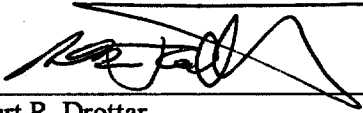
- 41 -

APPENDIX VI

-Continued-

Report Amendment

AMENDMENT SIGNATURES:

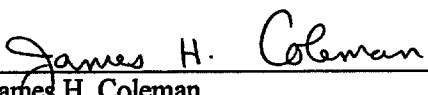

Kurt R. Drott
Study Director

4/26/00
DATE


Henry O. Krueger, Ph.D.
Director, Aquatic Toxicology and Non-Target Plants

4/26/00
DATE

REVIEWED BY:


James H. Coleman
Quality Assurance

4-26-00
DATE

000974

AMENDED

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: PFOS: A 96-HOUR STATIC ACUTE TOXICITY TEST WITH THE
SALTWATER MYSID (*Mysidopsis bahia*)

PROTOCOL NO.: 454/110998/MYS-96H1/SUB454

AMENDMENT NO.: 1

SPONSOR: 3M Corporation

PROJECT NO.: 454A-101

EFFECTIVE DATE: December 21, 1998

AMENDMENT: Page 2

Add: Experimental Start Date: 3/8/99

Experimental Termination Date: 3/12/99

Test Concentrations: Negative Control, 1.2, 1.9, 3.2, 5.4 and 9.0 mg a.i./L

Test Substance No.: 4675

REASON: The above information was not known when the protocol was signed by the Study Director.

AMENDMENT: Test Organism, Page 5

Delete: Feed provided to the mysids in the cultures will be analyzed at least once annually to ensure that there are no contaminants at levels known to be capable of interfering with the study.

REASON: Historical analyses of Wildlife International Ltd. aquatic feed have shown that no contaminants are present at levels known to be capable of interfering with the study.

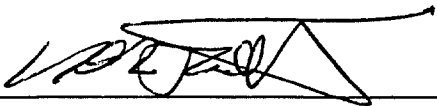
AMENDMENT: APPENDIX II, Page 13

Add: Liquid Chromatography Mass Spectrometry (LCMS) Method for the Determination of Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS) in Freshwater, Filtered Saltwater and Algal Medium.

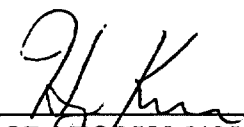
REASON: To add the analytical method to be used in the study.

000975

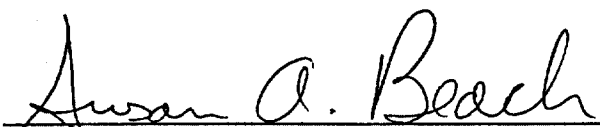
Reviewed by QA SLO 3-4-99


STUDY DIRECTOR

3/5/99
DATE


LABORATORY MANAGEMENT

3/5/99
DATE


SPONSOR'S REPRESENTATIVE

3/23/99
DATE

000976

ILDLIFE INTERNATIONAL LTD.

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: PFOS: A 96-HOUR STATIC ACUTE TOXICITY TEST WITH THE
SALTWATER MYSID (*Mysidopsis bahia*)

PROTOCOL NO.: 454/110998/MYS-96H1/SUB454

DEVIATION NO.: 1

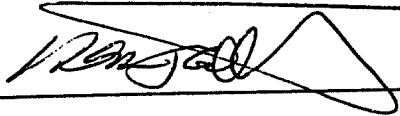
SPONSOR: 3M Corporation

PROJECT NO.: 454A-101

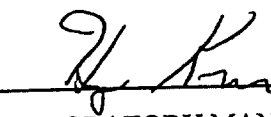
DATE OF DEFACTO DEVIATION: March 8, 1999

DEVIATION: The protocol states that the test chambers will consist of polyethylene bottles. The
test chambers actually consisted of plastic buckets.

REASON: Biologist oversight. It is the best judgment of the Study Director that this deviation did
not adversely affect the results of the study.


STUDY DIRECTOR

3/23/99
DATE


LABORATORY MANAGEMENT

3/23/99
DATE

WILDLIFE INTERNATIONAL LTD.

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: PFOS: A 96-HOUR STATIC ACUTE TOXICITY TEST WITH THE
SALTWATER MYSID (*Mysidopsis bahia*)

PROTOCOL NO.: 454/110998/MYS-96H1/SUB454

DEVIATION NO.: 2

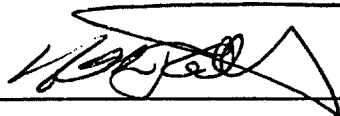
SPONSOR: 3M Corporation

PROJECT NO.: 454A-101

DATE OF DEFACTO DEVIATION: March 8, 1999

DEVIATION: The protocol states that measurements of pH will be measured in alternate replicates of each treatment and control group. Measurements of pH were actually made in all test chambers.

REASON: Biologist oversight. It is the best judgment of the Study Director that this deviation did not adversely affect the results of the study.



STUDY DIRECTOR

3/25/99

DATE



LABORATORY MANAGEMENT

3/25/99

DATE

PROTOCOL

PFOS: A 96-HOUR STATIC ACUTE TOXICITY TEST
WITH THE SALTWATER MYSID (*Mysidopsis bahia*)

U.S. Environmental Protection Agency
Series 850 - Ecological Effects Test Guidelines
OPPTS Number 850.1035

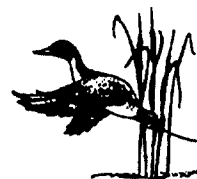
3M Lab Request No. U2723

Submitted to

3M Corporation
Environmental Laboratory
P.O. Box 33331
St. Paul, Minnesota 55133



WILDLIFE INTERNATIONAL LTD.



8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

November 9, 1998

000979

PFOS: A 96-HOUR STATIC ACUTE TOXICITY TEST
WITH THE SALTWATER MYSID (*Mysidopsis bahia*)

SPONSOR: 3M Corporation
Environmental Laboratory
P.O. Box 33331
St. Paul, Minnesota 55133

SPONSOR'S REPRESENTATIVE: Ms. Susan A. Beach

TESTING FACILITY: Wildlife International Ltd.
8598 Commerce Drive
Easton, Maryland 21601

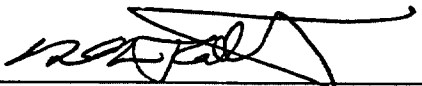
STUDY DIRECTOR: Kurt Drottar
Senior Aquatic Biologist

LABORATORY MANAGEMENT: Henry O. Krueger, Ph.D.
Director of Aquatic Toxicology & Non-Target Plants

FOR LABORATORY USE ONLY

Proposed Dates:	
Experimental Start Date: _____	Experimental Termination Date: _____
Project No.: <u>454A-101</u>	
Test Concentrations: _____	
Test Substance No.: _____ Reference Substance No. (if applicable): _____	

PROTOCOL APPROVAL



STUDY DIRECTOR

12/14/98

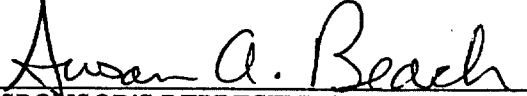
DATE



LABORATORY MANAGEMENT

12/14/98

DATE



SPONSOR'S REPRESENTATIVE

11/30/98

DATE

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INTRODUCTION

Wildlife International Ltd. will conduct a static acute toxicity test with the saltwater mysid (*Mysidopsis bahia*) for the Sponsor at the Wildlife International Ltd. aquatic toxicology facility in Easton, Maryland. The study will be performed based on the guidelines provided in the U.S. Environmental Protection Agency Series 850 - Ecological Effects Test Guidelines OPPTS Number 850.1035 (1); U.S. Environmental Protection Agency, Standard Evaluation Procedure, *Acute Toxicity Test for Estuarine and Marine Organisms* (2); and *ASTM Standard E729-88a Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3). Raw data for all work performed at Wildlife International Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International Ltd. site, or at an alternative location to be specified in the final report.

PURPOSE

The purpose of this study is to determine the acute effects of a test substance on the saltwater mysid (*Mysidopsis bahia*) during a 96-hour exposure period under static test conditions.

EXPERIMENTAL DESIGN

Saltwater mysids will be exposed to a geometric series of at least five test concentrations and a negative (dilution water) control for 96 hours. Two replicate test chambers will be maintained in each treatment and control group, with 10 mysids in each chamber for a total of 20 mysids per test concentration.

Nominal test concentrations will be selected in consultation with the Sponsor, and will be based upon the results of exploratory range-finding toxicity data, known toxicity data, physical/chemical properties of the test substance or other relevant information. Target concentrations need not exceed 1000 mg/L or the solubility limit of the test substance in water (whichever is lower). Generally, each test substance concentration used in the definitive test will be at least 60% of the next higher concentration unless information concerning the concentration-effect curve indicates that a different dilution factor would be more appropriate. Water samples from each test chamber will be collected at specified intervals for analysis of the test substance. Results of analyses will be used to calculate mean measured test concentrations.

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To control bias, juvenile mysids will be impartially assigned to exposure chambers at test initiation. No other potential sources of bias are expected to affect the results of the study. Each group of 10 will then be transferred to the test chamber. Observations of mortality and other clinical signs will be made throughout the 96-hour test period. Cumulative percent mortality observed in the treatment groups will be used to calculate, when possible, LC50 values at 24, 48, 72 and 96 hour intervals. The no-mortality concentration and the no-observed-effect concentration (NOEC) will be determined.

MATERIALS AND METHODS

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice Standards (GLP). The Sponsor is responsible for providing Wildlife International Ltd. written verification that the test substance has been characterized according to GLPs prior to its use in the study. If written verification of GLP test substance characterization is not provided to Wildlife International Ltd., it will be noted in the compliance statement of the final report. The attached form **IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR** (Appendix I) is to be used to provide information necessary for GLP compliance.

The Sponsor is responsible for all information related to the test substance and agrees to accept any unused test substance and/or test substance containers remaining at the end of the study.

Preparation of Test Concentrations

The test substance will administered to the test organism directly into dilution water. This route of administration was selected because it represents the most likely route of exposure to aquatic organisms.

Test Organism

The saltwater mysid (*Mysidopsis bahia*) has been selected as the test species for this study. Mysids are representative of an important group of aquatic marine invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory. Mysids to be used in the test will be less than 24 hours old and will be obtained from cultures maintained at Wildlife

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International Ltd., Easton, Maryland. The identity of the species will be verified by the supplier of the original culture or by Wildlife International Ltd. personnel using appropriate taxonomic keys such as Price (4).

Mysids will be cultured in water from the same source and at approximately the same temperature as will be used during the test. Mysids that show signs of disease or stress will not be used as test organisms.

Mysids in the cultures and during testing will be fed at least once daily. The diet will be live brine shrimp nauplii (*Artemia sp.*). Feeding during culturing may be supplemented with saltwater algae and rotifers as needed. Feed provided to mysids in the cultures will be analyzed at least once annually to ensure that there are no contaminants at levels known to be capable of interfering with the study. Specifications for acceptable levels of contaminants in mysid diets have not been established. However, there are no known levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the test.

Dilution Water

Natural seawater collected at Indian River Inlet, Delaware will be the source of the dilution water used in the culture facility and test system. The seawater will be filtered through a sand filter prior to its delivery to a 37,800-L holding tank. The salinity of the seawater will be diluted to approximately 20 ‰ (parts per thousand) with Wildlife International Ltd. well water in the holding tank. The 20 ‰ saltwater will be aerated using spray nozzles and filtered (0.45 μ m) prior to its delivery to the test system.

Salinity and pH will be measured weekly to monitor the consistency of the saltwater. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the seawater and results of the analyses will be summarized in the final report.

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Test Apparatus

Test chambers will consist of polyethylene bottles. The size of bottle and the volume of test solution will be sufficient to meet the requirements of sampling for analytical verification of test substance concentrations and routine water chemistry analyses. Test chambers will be indiscriminately positioned in a temperature-controlled water bath or an environmental chamber. Test chambers will be labelled with the project number, test concentration and replicate.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during holding and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent.

The target test temperature will be $25 \pm 2^{\circ}\text{C}$. Temperature will be measured to each test chamber at the beginning and end of the test using a liquid-in-glass thermometer. Temperature also will be measured with a continuous recorder in one negative control replicate. Recorder measurements will be verified with a liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen will be measured in all replicates of the treatment and control groups at test initiation and at approximately 24-hour intervals thereafter using a Yellow Springs Instrument Model 51B dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels fall below 60% saturation, appropriate actions will be taken after consultation with the Sponsor. Measurements of pH will be made in alternate replicates of each treatment and control group at test initiation and at approximately 24-hour intervals thereafter using a Fisher Accumet Model 915 pH meter, or equivalent. If a treatment group reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken at the next sampling interval, then discontinued. Dilution water salinity will be measured at test initiation using a Bio-Marine, Inc. Aquafauna refractometer, or equivalent.

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Biological Measurements

Observations of mortality and clinical signs of toxicity will be made between 0-24 hours, and at 24, 48, 72 and 96 hours $\pm$ 1 hour. The criteria for death include lack of movement, absence of respiratory movements, and lack of reaction to gentle prodding. Sublethal effects, such as abnormal locomotion, will also be noted. Dead organisms will be removed from the test chambers at each observation time.

Sampling for Analytical Measurements

Water samples will be collected from each test chamber at the beginning of the test, during the test, and at the end of the test to determine concentrations of the test substance. In the event that 100% mortality occurs in any treatment, then sampling of that treatment will terminate following the next sampling interval. Samples will be collected at mid-depth from each test chamber, analyzed immediately or placed in an appropriate storage container (e.g., polyethylene or polypropylene bottle) and stored under refrigeration until analyzed. The sample scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES			
Experimental Group	0 Hour	48 Hours	96 Hours
Control	2	2	2
Solvent Control (if needed)	2	2	2
Level 1-Low Concentration	2	2	2
Level 2	2	2	2
Level 3	2	2	2
Level 4	2	2	2
Level 5-High Concentration	2	2	2
Totals	14	14	14

Total Number of Verification Samples 42

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study.

Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International Ltd. The analytical method used will be based upon methodology provided by the Sponsor and identified in Appendix II. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Data Analysis

When the dose-response pattern allows calculation of an LC50 value, the data will be analyzed using the computer software of C.E. Stephan (5). The program was designed to calculate the LC50 value and the 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (6,7,8). The LC50 value will be calculated, when possible, using mortality data collected at 24, 48, 72 and 96 hours. The no-mortality concentration and the no-observed-effect concentration (NOEC) will be determined by visually interpreting the clinical observation data.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International Ltd. will include, but not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. Mysid history and culture records.
5. Stock solution calculation and preparation, if applicable.
6. Observations.
7. Water chemistry results (e.g., dissolved oxygen).
8. The methods used to analyze test substance concentrations and the results of analytical measurements.
9. Statistical calculations, if applicable.
10. Test conditions (light intensity, photoperiod, etc.).
11. Calculation and preparation of test concentrations.
12. Copy of final report.

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FINAL REPORT

A final report of the results of the study will be prepared by Wildlife International Ltd. The report will include, but not be limited to the following, when applicable:

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
7. A description of the methods used to conduct the test.
8. A description of the test organism, including the source, scientific name, age or life stage, feed types, light intensity and photoperiod.
9. A description of the preparation of the test solutions.
10. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers per treatment, and the duration of the test.
11. A description of circumstances that may have affected the quality or integrity of the data.
12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses.
14. Statistical methods used to evaluate the data.
15. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
16. The location where raw data and final report are to be stored.

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17. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
18. If it is necessary to make corrections or additions to a final report after it has been accepted, such changes will be made in the form of an amendment issued by the Study Director. The amendment will clearly identify the part of the final report that is being amended and the reasons for the amendment, and will be signed by the Study Director.

CHANGING OF PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and the Sponsor's Representative. Amendments will be considered as part of the protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to the protocol will be indicated in the final report.

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practice (OCDE/GD (92) 32, Environment Monograph No. 45); and Japan MAFF (59 NohSan, Notification No. 3850, Agricultural Production Bureau). Each study conducted by Wildlife International Ltd. is routinely examined by the Wildlife International Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International Ltd. site, or at an alternative location to be specified in the final report.

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REFERENCES

- 1 U.S. Environmental Protection Agency. 1996. Series 850- Ecological Effects Test Guidelines (draft), OPPTS Number 850.1035: *Mysid Acute Toxicity Test*.
- 2 U.S. Environmental Protection Agency. 1985. *Standard Evaluation Procedure, Acute Toxicity Test for Estuarine and Marine Organisms (Shrimp 96-Hour Acute Toxicity Test)*. Hazard Evaluation Division. Office of Pesticide Programs. EPA-540/9-85-010. Washington, DC.
- 3 ASTM Standard E729-88a 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians*. American Society for Testing and Materials.
- 4 Price, W.W. 1982. *Key to the Shallow Water Mysidacea of the Texas Coast with Notes on their Ecology*. Hydrobiologia 93, 9-21. Pr. W Juk Publishers, The Hague. Printed in The Netherlands.
- 5 Stephan, C.E. U.S. EPA. 1978. Environmental Research Laboratory, Duluth, Minnesota. Personal communication.
- 6 Finney, D.J. 1971. *Statistical Methods in Biological Assay*. Second edition. Griffin Press, London.
- 7 Thompson, W.R. 1947. *Bacteriological Reviews*. Vol. II, No. 2. Pp. 115-145.
- 8 Stephan, C.E. 1977. "Methods for Calculating an LC50," *Aquatic Toxicology and Hazard Evaluations*. American Society for Testing and Materials. Publication Number STP 634, pp 65-84.

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APPENDIX II

Analytical Method to be Provided by Sponsor

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